

AMENDMENTS TO THE SPECIFICATION

Please amend the specification as follows. Please replace the paragraphs beginning on p.8, line 12, with the following amended paragraphs.

Figure 10 shows serum IgG responses to lactate dehydrogenase-C (LDH-C) purified from tobacco leaves. The dark arrow represents 40 µg of plant LDH-C injected intraperitoneally. The lighter arrows are 20 µg boost. The red line is the date the animals were mated. The upper graph represents the average of a group of mice injected with pure lactate dehydrogenase-C emulsified in ~~Titermax~~-TITERMAX Gold or just the adjuvant as negative control. The lower graph represents the response of individual mouse from each group.

Figure 11 shows serum IgA responses to lactate dehydrogenase-C (LDH-C) purified from tobacco leaves. The upper graph represents the average of a group of mice injected with purified lactate dehydrogenase-C emulsified in ~~Titermax~~-TITERMAX Gold or just the adjuvant as negative control. The lower graph represents the response of individual mouse from each group.

Figure 12 shows vaginal IgG responses to lactate dehydrogenase-C (LDH-C) purified from tobacco leaves. The upper graph represents the average of a group of mice injected with purified LDH-C emulsified in ~~Titermax~~-TITERMAX Gold or just the adjuvant as negative control. The lower graph represents the response of individual mouse from each group.

Please replace the paragraph beginning on p.48, line 20, with the following amended paragraph:

Once enough quantities of lactate dehydrogenase-C were purified from plant material, the vaccine trials were performed. Six C57BL mice were used for this experiment. The choice of strain was based on fertility. This strain is very fertile having almost a dozen pups per litter. Forty µg of lactate dehydrogenase-C was emulsified in ~~Titermax~~-TITERMAX Gold® Adjuvant (Sigma, St. Luis, MO) and the formulation was injected into the intraperitoneal cavity of each mouse. As controls, ~~Titermax~~-TITERMAX Gold alone was emulsified in equal amounts of buffer and was injected into 4 mice. Three biweekly boosts of 20 µg lactate dehydrogenase-C emulsified in adjuvant followed until a substantial immune response was obtained. The animals were then mated and their fertility was tested. The immune response was tested on days -1, 6, 12, 19, 26, and 40. IgG and IgA isotypes were tested in serum and vaginal washes. The animals were mated on day 43 where several females were introduced into a cage containing a male previously assessed as fertile. The females were removed and placed in their cage after a clear vaginal plug was detected. Nineteen days after the first animal was impregnated, they were sacrificed and the number of fetuses per animal was counted.

Please replace the paragraph beginning on p.28, line 11, with the following amended paragraph:

After a plate with ~100 colonies was obtained from the cloning procedure, the cells were PCR screened for vectors containing the insert in the correct orientation. Since only one restriction enzyme was used, the gene could be inserted in the reverse direction. The colonies were PCR screened using the upstream primer LMV355Promoter (5'AGGACACGTGAAATCACCA) (SEQ ID No. 1) which is complementary to the Cauliflower Mosaic Virus Promoter and anneals to the vector. As a downstream primer the LDH-C3' was used (5'NNNNNGGATCCTACTATA ACTGCACATCCTTCTG) (SEQ ID No. 2). The new plasmid was called pLBJ21-LDH-C (Figure 4). The suspected positive colonies were used to inoculate a 10 ml LB culture with 15 µl of tetracycline (5 mg/ml) and the plasmid was isolated using the ~~Qiaprep~~-QIAPREP Spin Plasmid kit (Qiagen). The plasmids were submitted for sequencing to confirm the correct lactate dehydrogenase-C sequence was contained in pLBJ21-LDH-C.

Please replace the paragraph beginning on p. 30, line 2, with the following amended paragraph:

The cloning of the SPV into the plant expression vector pLBJ21 was similar to the cloning of pLBJ21-LDH-C described above. Plasmid pcDNA3.1-SPV was subjected to restriction digest using EcoRI. After digestion, the gene was cloned into digested pLBJ21. Successful cloning was confirmed by PCR screening. The new plasmid was called pLBJ21-SPV (Figure 6). A few colonies from positive cloning were selected and a plasmid preparation was done using the ~~Qiaprep~~-QIAPREP Spin Plasmid kit. The plasmids were subjected to sequencing using the CaMV primer described above.

Please replace the paragraph beginning on p.30, line 12, with the following amended paragraph:

Strain GV3101(pMP90) of *Agrobacterium* (Knocz and Schell, 1986) was frozen in LB media supplemented with 10% glycerol. From this glycerol stock a 5 ml culture was inoculated and grown overnight in a 30°C incubator shaking at 200 rpms. A dense culture was obtained next morning and was used to make a stock of electrocompetent *Agrobacterium*. One µl of each plasmid (approximately 50 ng) was mixed with 20 µl of freshly prepared cells and the mixture was incubated on ice for 30 minutes. The cells were transferred to an electroporation chamber Micro Electro Chamber (Gibco BRL Life Technologies). The chambers were placed in the ~~Cell Porator~~-CELL PORATOR Electroporation System I (Gibco BRL Life Technologies) and the transformation was performed according to the manufacturer's instructions.

Please replace the paragraph beginning on p.30, line 22, with the following amended paragraph:

The transformed *Agrobacterium* were recovered in 250 µl of SOC media at 30°C while shaking at 200 rpm for 1 hour. The cells were further plated in double selection LB agar plates containing 50 mg/L kanamycin and gentamycin. The plates were incubated at 30°C for 48 hours. About 10 colonies from each plate were PCR-screened. PCR was performed in a Perking Elmer ~~Gene Amp~~ GENE AMP PCR system 2400 using the following temperature sequence: 5 minutes, 94°C; 30 cycles of 45 seconds, 94°C, 45 seconds, 55°C, 1.5 minutes, 72°C; 10 minutes, 72°C. After PCR, positive clones were visualized on agarose gel. It is important to run a positive control (the original insert) and a negative control (water) so bands in the gel can be compared accordingly. Successful transformations were kept at -80°C in glycerol stocks until further use.

Please replace the paragraph beginning on p. 33, line 4, with the following amended paragraph:

The seeds were ready for selection at this point and they were sterilized by placing them in a folded piece of filter paper closed accordingly to make a “tea bag”. The filter paper was submerged in a solution of 20% bleach with 0.1% ~~tween~~ TWEEN 20 (polysorbate, Sigma) for 15 minutes. They were washed afterwards 3 times with distilled water. After the seeds were sterilized they were placed and distributed evenly in GM plates containing 100mg/L of kanamycin (Sigma) and timetin (SmithKline Beecham) under sterile conditions. The GM plates were composed of 0.5 X Murashige and Skoog salt mixture (Sigma), 1% sucrose, MES pH 5.7 (Sigma), and 0.8% tissue culture grade agar (Sigma). The plates were kept sealed with Parafilm at 4°C in the dark for 2 days. On the third day, the plates were moved to an incubator at 22°C with a 24-hour light cycle.

Please replace the paragraph beginning on p. 44, line 6, with the following amended paragraph.

Production of specific antibodies against lactate dehydrogenase-C in serum and vaginal washes was assessed by indirect ELISA. Briefly, 50 µl of 0.01 mg/ml recombinant lactate dehydrogenase-C in capture buffer (100 mM sodium bicarbonate, pH 9.2) was coated onto 96-wells microtiter plate (Flow Laboratories, McLean VA) and were further incubated overnight at 4°C. The plates were washed 4 times with PBST (phosphate buffer saline, pH 7.4, 0.5% ~~tween~~ TWEEN 20 (polysorbate, Sigma)), incubated at room temperature in blocking buffer (PBST with 1% protease-free BSA (Roche Biochemicals)) for 30 minutes and washed again once. Serum or vaginal washes were added to the wells up to 50 µl in blocking buffer and the plates were incubated for 1 hour at room temperature. When IgA was tested both serum and vaginal washes were diluted 1:10. When IgG was tested the serum was diluted 1:500 and vaginal washes were diluted 1:10. The plates were washed 3 times as described above and then a secondary affinity purified goat anti- mouse IgG or goat anti-mouse IgA coupled to a horseradish peroxidase was

added. Both antibodies were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). For IgG assessment, the antibodies were used at 1:10,000 dilution in blocking buffer. For IgA, a 1:1000 dilution was used. After one hour incubation at room temperature the plates were washed 4 times and 100 µl of hydrogen peroxide-2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) from Moss Inc. (Pasadena, MD) was added and incubated for 30 minutes in the dark. The reaction was quenched with 100 µl of 0.5 M oxalic acid. The plates were immediately read at 414 nm in an EL340 Automated Microplate Reader (Bio-Tek Instruments Inc., Winooski VM).

Please replace the paragraph beginning on p. 39, line 13, with the following amended paragraph:

A small sample of transgenic or wild type tobacco leaf tissue (~100 mg) was crushed with a mortar and pestle in the presence of liquid nitrogen. The pulverized tobacco material was transferred to a 1 ml glass tissue grinder (Corning) at 4°C. Immediately thereafter, 500 µl of pre-chilled extraction buffer were added (PBS supplemented with 2 mM PMSF, 2X Complete Protease Inhibitor Cocktail (Roche Biochemicals) and 2 mM EDTA). The tobacco was ground until a homogenous solution was obtained. The solution was transferred to a 2 ml microtube and was centrifuged in an ~~Eppendorf~~-EPPENDORF Centrifuge 5417C (Eppendorf) at maximum speed for 20 minutes at 4°C. The supernatant was transferred to a fresh tube and the protein concentration was determined by the Warburg-Christian method. All samples to be tested including the wild type sample were normalized to a common protein concentration by dilution with phosphate buffered saline (PBS) such that accurate measurement of lactate dehydrogenase-C (LDH-C) could be performed.

Please replace the paragraph beginning on p. 42, line 5, with the following amended paragraph.

The next morning, the dialyzed 40-80 AS fraction was placed in a pre-set ultrafiltration apparatus containing a ~~Diaflo~~-DIAFLO membrane XM300 (Amicon, Beverly, MA). The sample was concentrated to about 5 ml. The extract was adjusted to 5% sodium bicarbonate and a few mg of sucrose was added to sweeten the plant extract thereby making it more palatable. The protein concentration was quantified and normalized to the wild type concentration such that equal amounts of protein would be given in extracts from transgenic plants or controls. The samples were aliquoted into small test tubes and stored at -80°C until further use. The amount of lactate dehydrogenase-C contained in each extract was quantified using the sandwich ELISA developed as described in the previous section.

Please replace the paragraph beginning on p. 46, line 21, with the following amended paragraph.

Four hundred grams of tobacco leaf tissues were freshly harvested right before extraction. Leaves were homogenized in a commercial juicer at 4°C and from this moment the sample was

always kept at this temperature, unless stated otherwise. The pulp was juiced at least twice and then it was discarded. The homogenate was supplemented with 10X extraction buffer [100 mM phosphate buffer pH 7.0, 10 mM ethyldiaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), 10 mM β -mercaptoethanol (BME), 10 mM phenyl methyl sulfonyl fluoride, 2 tablets/30 ml of Complete Protease Inhibitor Cocktail[®] (Roche Biochemical, Germany)]. The sample was centrifuged for 30 minutes at 18,000 rpm in a ~~Sorvall~~-SORVALL SS-34 rotor (Du Pont, Wilmington, DL). The sample was filtered through a ~~Whatman~~-WHATMAN 158mm filter (Whatman, England) and then transferred to a centrifuge tube.